Trends in Plant Science

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Review

Revisiting the Female Germline and Its Expanding Toolbox

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The *Arabidopsis thaliana* ovule arises as a female reproductive organ composed solely of somatic diploid cells. Among them, one cell will acquire a unique identity and initiate female germline development. In this review we explore the complex network that facilitates differentiation of this single cell, and consider how it becomes committed to a distinct developmental program. We highlight recent progress towards understanding the role of intercellular communication, cell competency, and cell-cycle regulation in the ovule primordium, and we discuss the possibility that distinct pathways restrict germline development at different stages. Importantly, these recent findings suggest a renaissance in plant ovule research, restoring the female germline as an attractive model to study cell communication and cell fate establishment in multicellular organs.

Highlights

In the plant ovule, a single cell of somatic origin initiates a developmental program distinct from that of adjoining cells.

This cell, typically known as the megaspore mother cell (MMC), is also referred to as the primary female germline cell.

Rapid advances in cell type-specific profiling technologies, deep-tissue microscopy, and marker gene resources have reinvigorated the study of female germline development.

Key results show that epigenetic pathways, cell-cycle regulators, and mobile signals act to establish and restrict female germline identity.

Recent reports detail how several of these pathways are interconnected.

The Basics of Ovule Development and Germline Initiation in Plants

The vast majority of flowering plants reproduce sexually, following a life cycle that alternates between diploid (sporophytic/somatic) and haploid (gametophytic) generations. This alternation is a fundamental requirement for plant speciation because it leads to the production of genetically distinct gametes containing new combinations of alleles, facilitates their dissemination, and provides an environment for protection and nourishment [1]. The sporophytic phase of plant development is dominated by the growth of organs such as leaves, stems, branches, and eventually flowers (Figure 1A) [2]. Deep inside the flowers the gametophytic phase prepares to appear, beginning with the formation of haploid spores in specialized reproductive organs called ovules (Figure 1B). Depending on the *Arabidopsis thaliana* (arabidopsis) accession, ~40–80 finger-like ovules composed solely of diploid cells will emerge from a placental tissue located at the junction of two fused carpels (Figure 1B) [3]. Divisions of the diploid ovule cells give rise to three prominent domains along a proximal–distal axis (Figure 1C). The proximal funiculus acts as a stalk to connect the ovule to the placenta and maternal plant, and the central chalaza gives rise to the integuments and seed coat. The tip of the ovule, referred to as the **nucellus** (see Glossary), produces a single germline cell (**megaspore mother cell, MMC**) that is the progenitor of a single haploid **functional megaspore** (FM) and a single **female gametophyte** (FG) (Figure 2).

In arabidopsis, similarly to most angiosperms, the position of the single germline precursor is precisely controlled. Early in ovule development, two layers (L) of nucellus can be distinguished – including an epidermal layer (L1) and a subepidermal layer (L2; Figures 1C and 2). The L2 functions as a sporogenous tissue and fills the nucellar dome. In arabidopsis, the most distal L2 cell will become the germline, first via differentiation into an **archesporial cell** (AC) and then into the MMC [4]. This stage of germline development is also referred to as **megasporogenesis**. Although the concept of a true plant germline has been contentious, the term is now routinely used; the different stages are discussed in Box 1. **Germline initiation** is

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characterized by an increase in AC/MMC volume [5], changes in cell wall composition, histone modifications, and nucleosome remodeling [6], followed by meiosis and the production of four haploid megaspores [4] (Figure 2 and Box 2). The FM is the chalazal-most megaspore, the sole survivor after meiosis, and shows molecular and chemical features that are distinct from the MMC and surrounding cells (Box 2) as it transitions to a **germline maturation** phase. Also known as megagametogenesis, this phase involves three rounds of mitosis and leads to the production of an embryo sac, also known as the megagametophyte or FG [7], that contains the mature **female gamete** (egg cell; Figure 2) ([8] for recent review).

Early genetic studies of ovule development proposed that development of the single post-meiotic germline cell (i.e., the FM) depends on information from surrounding diploid cells. In the absence of one or more genes essential for integument development, such as *AINTEGUMENTA* (*ANT*) [9], *INNER NO OUTER* (*INO*) [10], *BELL1* [11–13], *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*), *SHP2* [12,13], and *ARABIDOPSIS B SISTER* (*ABS*) [14], FG maturation is impaired. This was reinforced by later studies that implicate genes expressed in the nucellar epidermis in the control of FG development [15,16]. The evidence for regional communication during germline maturation appears to be strong because most of these genes are not expressed in the haploid germline lineage despite influencing its development [10,15–19].

Whether similar communication pathways contribute to the initiation of the **female germline lineage**, before the appearance of the integuments, has proved far more challenging to address. Indeed, relatively few factors influencing female germline initiation have been identified other than the classical nuclear protein NOZZLE/SPOROCYTELESS (NZZ/SPL), which acts in concert with the homeodomain transcription factor (TF) WUSCHEL (WUS; Figure 1). *nzz/spl* mutants fail to produce a germline in the majority (99%) of ovules [20], while *wus* mutants lack a primary germline cell in ~12% of ovules [21]. Both WUS and NZZ/SPL are expressed in the nucellus well before germline initiation, suggesting that, at least initially, they may act **cell-autonomously** to establish an environment for germline formation. However, after MMC formation their expression becomes restricted to non-germline cells, suggesting that they may also function to distinguish germline from somatic identity.

Our objective in this review is to revisit the cell-autonomous and **non-cell-autonomous** control of early female germline development, and to formulate a mechanistic model summarizing progress to date. Additional components include epigenetic pathways that control **germline identity** ([15,22–28]; [29] for relevant review), cell-cycle regulators that facilitate correct progression of a germline program [30–32], and hormones that provide an environment supporting sporogenic potential [27,33]. Although it is not always clear how these components are integrated, recent technological improvements in precision phenotyping, mutagenesis, and transcriptomics provide clear opportunities to test this model in the future (Figure 1D).

Establishment of Germline Identity in the Ovule Requires a Raft of Epigenetic, Possibly Non-Cell-Autonomous, Pathways

In simple terms, the initiation of the female germline in arabidopsis begins with specification of a subepidermal L2 cell as the germline precursor, enlargement of that cell to form the MMC, and the initiation of meiosis. Once the MMC begins to expand, its epigenetic state quickly becomes distinct from that of the surrounding tissues: the chromatin is less condensed, there is a reduction in heterochromatin, and the core histone variants change [24]. This matter has been elegantly discussed in a recent review [6]. Several epigenetic pathways appear to enforce the distinction between **germline fate** and somatic fate. For example, ARGONAUTE 9 (AGO9), a component of the RNA-directed DNA methylation (RdDM) machinery, utilizes 24 nt small RNAs

Glossary

Apomixis: a form of asexual reproduction that results in clonal maternal progeny through seed.
Apospory: a mode of apomictic reproduction where at least one unreduced female gametophyte (FG) is produced from a somatic cell adjoining the megaspore mother cell (MMC) or functional megaspore (FM).
Archesporial cell (AC): precursor cell of the female germline and progenitor of the MMC.
Cell-autonomous: a mechanism that occurs in a cell and produces effects on that cell.
Diplospory: a mode of apomictic reproduction where an unreduced female gametophyte is produced directly from an MMC that avoids meiosis.
Female gamete: the egg cell, which is located in the female gametophyte.
Female gametophyte (FG): a haploid structure produced by divisions of the FM, also known as the embryo sac. The mature gametophyte contains four different cell types, one of which is the egg cell that functions as the female gamete.
Female germline lineage: the cellular lineage originating from the diploid MMC and that gives rise to a haploid FM, ending with the formation of a mature female gamete.
Functional megaspore (FM): the only megaspore out of four meiotic products that survives, enters megagametogenesis, and forms the FG.
Germline fate: refers to cells that adopt a germline developmental program.
Germline identity: cells including the AC, MMC, FM, and FG, as well as abnormal nucellar cells from mutants where cellular identity has been compromised in the ovule.
Germline initiation: formation of an AC and its differentiation into the primary germline cell, the MMC.
Germline maturation: defines the stages during which the FM undergoes a series of mitotic divisions and cellularization to form the FG.
Megagametogenesis: describes the processes occurring from FM

(sRNAs) to silence transposable elements in the nucellus (Figure 1D) [15,34]. In *ago9-3* mutants, ovules exhibit an increased number of enlarged subepidermal cells, which acquire FG identity without undergoing meiosis (Figure 3) [15]. RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) functions in the biogenesis of *trans*-acting small interfering RNAs (tasiRNAs; Figure 1D) [34], and *rdr6-11* mutant ovules show a similar phenotype to *ago9* (Figure 3) [15]. Models suggest that the AGO9/RDR6 pathway involves gene silencing, thereby restricting multiple nucellar cells from expanding and adopting FG identity [15]. Interestingly, the enlarged nucellar cells in *ago9* and *rdr6* mutants exhibit histone modifications similar to those of the MMC [24], suggesting they may share some mixed MMC/FG identity. Another factor that appears to play a similar role is MNEME (MEM), a putative ATP-dependent RNA helicase that is specifically expressed in the MMC (Figure 1D) [35]. Mutations in *MEM* result in the formation of multiple enlarged germline-like cells in the nucellus (Figure 3) and, occasionally, unreduced FGs that exhibit altered epigenetic marks. This suggests a potential role for MEM in restricting germline identity in some somatic cells and in establishing a germline-specific chromatin state (Figure 1D). How this role in regulating somatic cell identity might be achieved via specific expression of *MEM* in the germline itself is currently unclear.

The location of AGO9 protein is also of key importance based on its function in restricting germline identity. Initial immunolabeling studies suggested that AGO9 protein was restricted to the L1 of the nucellus [15], supporting a non-cell-autonomous function in underlying nucellar cells. However, subsequent studies showed AGO9 protein within the nucleus of the MMC and cytoplasm of adjoining L2 ovule cells, in addition to the L1 [36]. Hence a balance between cell-autonomous and non-cell-autonomous functions seems possible (Figure 1D). During spermatogenesis in *Drosophila* testis, the germline cells develop while surrounded by cyst cells of somatic origin [37], conceptually similar to what takes place in the plant ovule. The PIWI protein, an animal-specific member of the AGO protein family, is detected in both cyst and germline cells; however, PIWI expression in the cyst cells leads to differentiation of the germline cells in a non-cell-autonomous fashion [38]. Given that PIWI proteins in animals and at least one AGO in arabidopsis (e.g., AGO10 [39]) contribute to non-cell-autonomous pathways during development, the cell-specific requirement for AGO9 appears to warrant further investigation via transgenic cell type-specific expression studies. Differential function in different cell types could potentially relate to where distinct sRNAs accumulate or where gene silencing partners are expressed. AGO9 can bind to a range of sRNA molecules [34], but whether only one specific class facilitates its function in repressing the enlargement of multiple subepidermal cells remains uncertain. Silencing partners are likely to include other members of the RdDM pathway because mutations in *AGO4*, *AGO6*, *AGO8* [40], *RDR2*, *SGS3*, *DCL3*, and *NRPD1a/b* [15] also induce a phenotype resembling that observed for *ago9* or *rdr6* (Figure 3) (reviewed in [41]).

Although the genic targets of the AGO9/RDR6 RdDM pathway have proved elusive, recent studies show that RDR6 functions with TEX1, a component of the tasiRNA biogenesis machinery [42]. This pathway leads to the production of *TAS3*-derived tasiRNA, which represses expression of the AUXIN-RESPONSIVE FACTOR 3 (ARF3) TF in the ovule (Figure 1D) [27]. ARF3 plays a key role in integument growth [43] as well as in floral meristem development where it is involved in repression of *WUS* [44]. *tex1*, *tas3*, and *rdr6* accumulate increased levels of *ARF3* transcript; furthermore, in *tex1* and in a *TAS3*-resistant *ARF3* mutant, the *ARF3* expression domain extends beyond the chalaza into the nucellar epidermis. The deregulation of *ARF3* is associated with the presence of supernumerary MMCs that sometimes develop into unreduced FGs (Figure 3) [27]. Although the origin of the abnormal germline-like cells was not determined, the ovules typically produced only one extra MMC [27], which contrasts with mutants where multiple MMCs originate from abnormal MMC mitosis (Figure 3

expansion until the production of a mature FG.
Megaspore: one of four haploid cells resulting from meiosis of the MMC.
Megaspore mother cell (MMC): the most distal cell within the L2 of the nucellus that acts as the primary germline cell.
Megasporogenesis: a term describing the processes occurring from MMC formation until the production of a FM.
Non-cell-autonomous: a mechanism that occurs in one cell but produces effects on another cell.
Nucellus: distal ovule tissue composed of diploid cells organized into two layers (L), including the L1 epidermal cells and L2 hypodermal cells, where the germline cell lineage will develop.

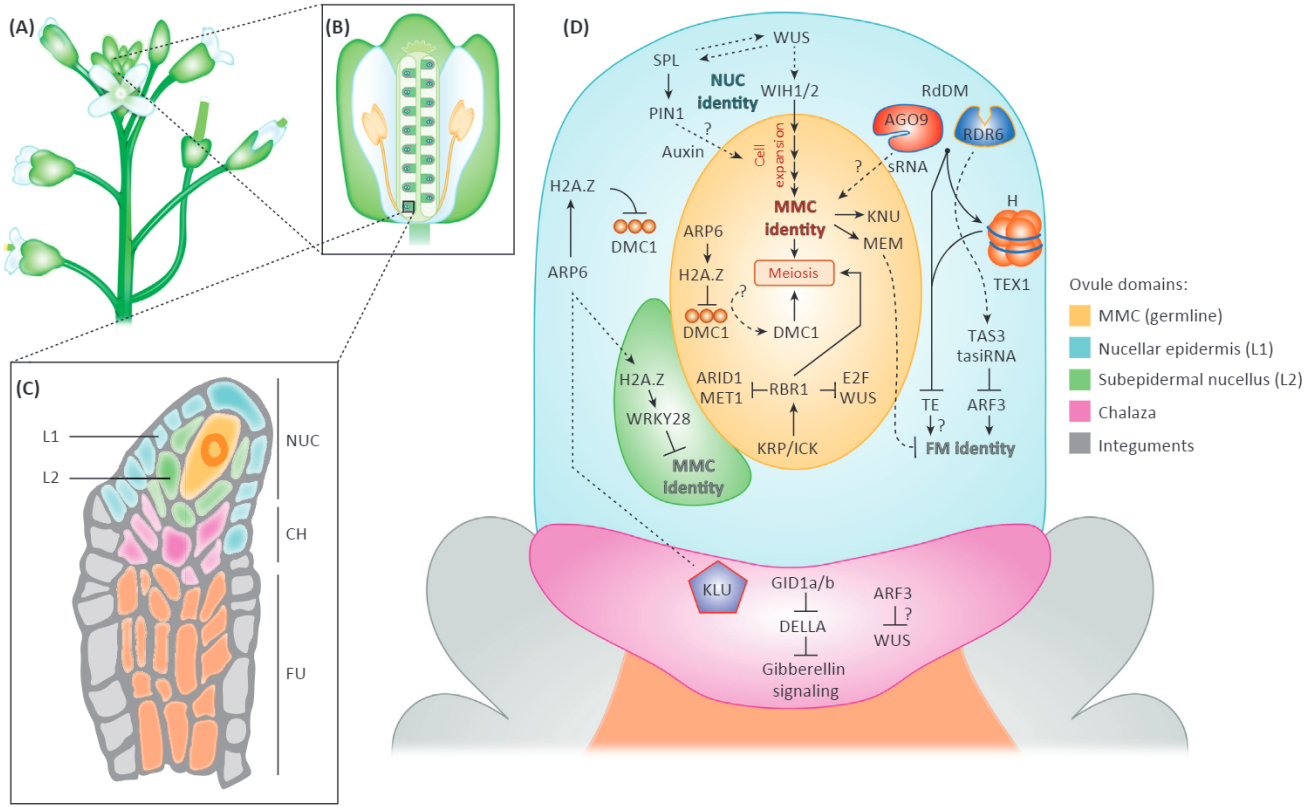

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Figure 1. Schematic Representation of Key Pathways Implicated in Female Germline Development. (A) Graphical representation of an *Arabidopsis thaliana* (arabidopsis) inflorescence showing flowers at different developmental stages, and (B) a young floral bud showing the location of immature ovules within the developing carpels. (C) Schematic diagram of a young arabidopsis ovule containing a single primary germline cell, the megaspore mother cell (MMC). The location of the nucellus (NUC), chalaza (CH), and funiculus (FU), as well as layer 1 (L1) and L2 cells in the distal domain are indicated. (D) Model integrating pathways involved in female germline development. Although the precise relationship is unclear, SPOROCYTELESS (SPL) and WUSCHEL (WUS) act in the nucellus to drive expression of *PIN-FORMED1* (*PIN1*) and a set of corepressors [73–75], establishing an environment required for germline initiation. WUS function in promoting MMC formation is possibly mediated via the *WINDHOSE 1* (*WIH1*) and *WIH2* genes [21]. WUS, SPL, and WIH1/2 act as markers of nucellar identity in the ovule. After germline specification, the MMC rapidly expands [5], although the mechanistic basis for this has yet to be revealed. The KNUCKLES (KNU), MNEME (MEM), and ARGONAUTE 9 (AGO9) proteins are expressed in the MMC, but their specific functions within the germline are unknown [16,27,35]. Also in the MMC, ACTIN-RELATED PROTEIN 6 (ARP6) promotes the deposition of H2A.Z histone variant in the *DISRUPTION OF MEIOTIC CONTROL 1* (*DMC1*) gene body. An unknown developmental stimulus promotes expression of *DMC1*, leading to initiation of MMC meiosis. The same pathway acts in the nucellus, but the absence of the stimulus prevents *DMC1* from functioning in non-sporogenous cells [25]. For the MMC to enter meiosis, RETINOBLASTOMA-RELATED 1 (RBR1) represses *WUS* expression [30], and possibly *AT-RICH INTERACTING DOMAIN 1* (*ARID1*) and *DNA METHYLTRANSFERASE 1* (*MET1*) [26]. As part of the RNA-directed DNA-methylation pathway (RdDM), AGO9, RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and other components participate in the biogenesis of small RNAs (sRNAs) and appear to be involved in histone modifications (H) which silence transposable elements (TEs) and restrict the acquisition of functional megaspore (FM) identity in the nucellar cells [15]. RDR6 and TEX1 are implicated in the formation of *TAS3*-derived *trans*-acting short interfering RNA (tasiRNA). This class of tasiRNA represses *AUXIN RESPONSIVE FACTOR 3* (*ARF3*), a negative regulator of MMC identity [27]. ARF3 is expressed in central regions of the ovule where it may be involved in restricting *WUS* expression, similarly to its role in the floral meristem [44]. Expression of GIBBERELLIN-INSENSITIVE DWARF 1 (GID1) is also restricted to this domain where it mediates gibberellin-dependent signaling pathways. Expansion of *GID1a* expression into the nucellus appears to alter the size of L2 nucellar cells. Finally, KLUH (KLU) interacts with ARP6 via the chalaza to promote H2A.Z deposition at the *WRKY28* locus. This causes specific *WRKY28* expression in the subepidermal nucellar cells, preventing the acquisition of MMC identity [28]. Broken arrows represent putative processes and question marks indicate unknown factors/mechanisms that remain to be identified. The scheme is not drawn to scale and color coding is indicated on the right.

and below). Therefore, it is possible that the phenotype is caused by a subtle shift in subepidermal nucellar identity towards a germline identity [27], dependent upon aberrant *ARF3* expression in the nucellar epidermis, while the original MMC maintains a normal developmental program.

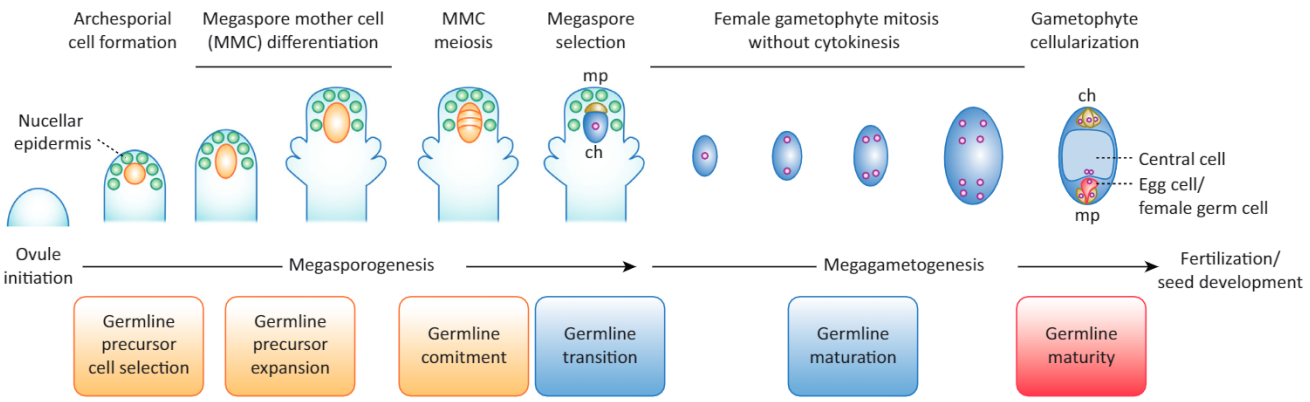

Figure 2. Female Germline Development in *Arabidopsis thaliana* (arabidopsis). After ovule initiation, the key events in female germline development take place in the distal domain of the ovule. The first visual indication of germline development begins with selection of a diploid archesporial cell (AC) from a pool of subepidermal L2 cell marks. This cell directly adjoins the nucellar epidermis, a single layer of cells surrounding the nucellus, which are indicated with green nuclear dots in the figure. In arabidopsis the AC acts as the germline precursor and expands to directly form the megaspore mother cell (MMC). The MMC is the primary germline cell. The initiation of meiosis effectively commits the MMC to the downstream stages of germline development, and selection of a single meiotic product (the functional megaspore, FM) sees germline development transition to a haploid phase. The subsequent stages of germline development occur within this cell, which divides without cytokinesis to form a mature multicellular gametophyte containing a single egg cell, the female germ cell. Double fertilization of the egg cell and central cell cue the downstream stages of seed development. Abbreviations: ch, chalaza; mp, micropyle.

Chromatin Remodeling Provides an Additional Checkpoint To Support Germline Entry into Meiosis

Female germline identity is usually associated with a unique capacity to enter meiosis, suggesting that mechanisms are in place to restrict this to the single germline cell. ACTIN-RELATED PROTEIN 6 (ARP6) is part of a chromatin remodeling complex that mediates the deposition of H2A.Z histone variant into nucleosomes [45] and acts to distinguish MMC identity. In arabidopsis, *ARP6* is expressed in the MMC and surrounding ovule cells and represses *DISRUPTION OF MEIOTIC CONTROL 1* (*DMC1*) by mediating preferential deposition of H2A.Z in the *DMC1* gene body (Figure 1D). These marks also leave *DMC1* in a potentially 'responsive' state. Subsequent developmental stimuli that cue the onset of meiosis can remove histone variants in the MMC, allowing *DMC1* transcription. In *arp6* mutants, reduced deposition of H2A.

Box 1. Defining the Plant Female Germline

The concept of a true germline in plants is controversial because, unlike animals that separate a germline from the soma early in development, plants initiate gamete production much later in their life cycle from cells that previously had no fixed reproductive identity ([67] for review). As a result, most plant biologists have refrained from using the term 'germline development', preferring more classical botanical terms such as megasporogenesis and megagametogenesis to describe the cellular events leading to gamete formation (see Figure 1 in main text). However, over the past 20 years the language in the plant development field has noticeably changed. Studies of male reproductive development, such as those from Custers [76], Sorenson [77], and Rotman [78], discussed the 'male germline', and it has now become far more commonplace to use this terminology in the context of both male and female reproduction ([79] for an earlier review). In general, this change appears to be beneficial for the development community because it facilitates simpler plant-versus-animal comparisons that appeal to a broader scientific audience. Unfortunately, the assortment of terms available to describe female development causes confusion. For example, what is the primary female germline cell – the AC, MMC, FM, or FG? In Figure 2, main text, we present a schematic diagram that defines several stages of germline development, aligning them with more classical reproductive stages. The figure highlights that female germline development begins with the formation of a germline precursor cell (the AC/MMC) that commits to germline development upon entering meiosis. The selected product of meiotic division (the FM) transitions to a germline maturation phase before finally giving rise to the female gamete itself, the egg cell. Thus, as it prepares to enter meiosis, the MMC is considered to be the primary germline cell.

Box 2. To Be or Not To Be an MMC, That Is the Question

The appearance of multiple enlarged germline-like cells in the nucellus of arabidopsis ovule mutants inevitably raises questions regarding cell identity. Are the extra cells megaspore mother cells, FMs, or something else altogether?

Clearing of arabidopsis ovules with Hoyer's solution followed by differential interference contrast (DIC) microscopy provides information regarding the presence of enlarged cells in the nucellus because the nuclei and wall of each cell can be discerned. The appearance of multiple enlarged cells can be also be confirmed by more labor-intensive confocal imaging [5] combined with reconstructive modeling via MorphoGraphX [80]. However, it is not possible to use these techniques in isolation to draw conclusions regarding cell identity. Several of the reported 'extra germline cell' phenotypes are subtle; whether this is a result of variable growth conditions, gradual changes in phenotypic penetrance, or a lack of expertise is not known, and additional evidence is required.

Marker genes can be useful to distinguish germline cells from surrounding tissues. Regulatory elements within the *KNUCKLES* (*KNU*) gene are sufficient to drive expression in the primary germline cell (the MMC), hence fusions to GUS or fluorophores act as excellent markers for MMC identity [16,71]. Some care must be taken when the MMC enters meiosis, however, because *pKNU*-driven transcriptional reporters remain expressed in the megaspore tetrad. Another marker for the female germline is *pDMC1:GFP*, which accumulates in the germline once meiosis has initiated [48]. In principle, true multi-MMC mutants would be expected to show multiple cells expressing a *pKNU:YFP* reporter during pre-meiotic stages, and then *pDMC1:GFP* if they enter meiosis.

After meiosis, markers of the FM and/or young FG such as *pFM1:GUS* [72], *pFM2:GUS* [15], *pLC2:nlsYFP* [16,81], and *pSUF4::SUF4–GUS* [82] only switch on in the FM. In principle, mutants producing additional FM or FG cells might be expected to show multiple cells expressing these identity markers.

In terms of diagnostic morphological information, callose is a classical marker of germline cells in reproductive tissues. Callose is weakly detected in the MMC wall by aniline blue staining [83], becomes prominent in the walls of the meiotic dyad and tetrad, but is lacking in the FM. By contrast, anti-AGP antibodies such as JIM13 show prominent labeling in the FM wall after megaspore selection, whereas the wall of the arabidopsis MMC appears to lack the corresponding epitope [84,85]. Hence, the distinction between MMC and FM cell wall composition might also be considered when addressing the identity of additional enlarged cells.

Z in *DMC1* leads to ectopic expression of *DMC1* in somatic cells surrounding the MMC and impairs MMC meiosis. Hence, ARP6 is crucial for *DMC1* repression in the somatic cells of the ovule (Figure 1D) [25]. It is intriguing, however, that ectopic *DMC1* expression in the nucellar cells does not appear to trigger ectopic meiotic divisions. This highlights the existence of additional molecular checkpoints that provide the competency to enter meiosis. Because *DMC1* is maintained in a responsive state in nucellar cells surrounding the MMC, Qin *et al.* [25] proposed that the ovule maintains a degree of epigenetic plasticity to allow the development of a second germline precursor in the case that MMC differentiation fails. Supporting this notion is the fact that similar chromatin modifications are present in the MMC and adjoining nucellar cells in maize [23]. In addition, the *AGO* gene *MEIOSIS ARRESTED AT LEPTOTENE 1* (*MEL1*) in rice is initially expressed in numerous archespores in the ovule primordium; however, only one archespore will initiate germline development, and *MEL1* expression becomes restricted to that cell [22].

An additional factor that acts with ARP6 is KLUH (KLU), a cytochrome P450 monooxygenase. KLU controls cell plate formation during megasporogenesis, presumably through the production of a mobile signal that diffuses from the base of the inner integument to adjoining tissues (Figure 1D) [46–48]. Moreover, KLU acts with ARP6 to repress germline identity in the subepidermal nucellar cells [28]. In the *klu arp6* double mutant, ovules present supernumerary MMC-like cells that remain in a quiescent state (Figure 3). WRKY28, a zinc-finger WRKY TF, was shown to be significantly downregulated in *klu arp6*, while *wrky28-Cas9-3* ovules phenocopy the *klu arp6* double mutant (Figure 3) [28]. In contrast to *KLU*, *WRKY28* is expressed in the L2 cells surrounding the MMC. Remarkably, chromatin immunoprecipitation (ChIP)

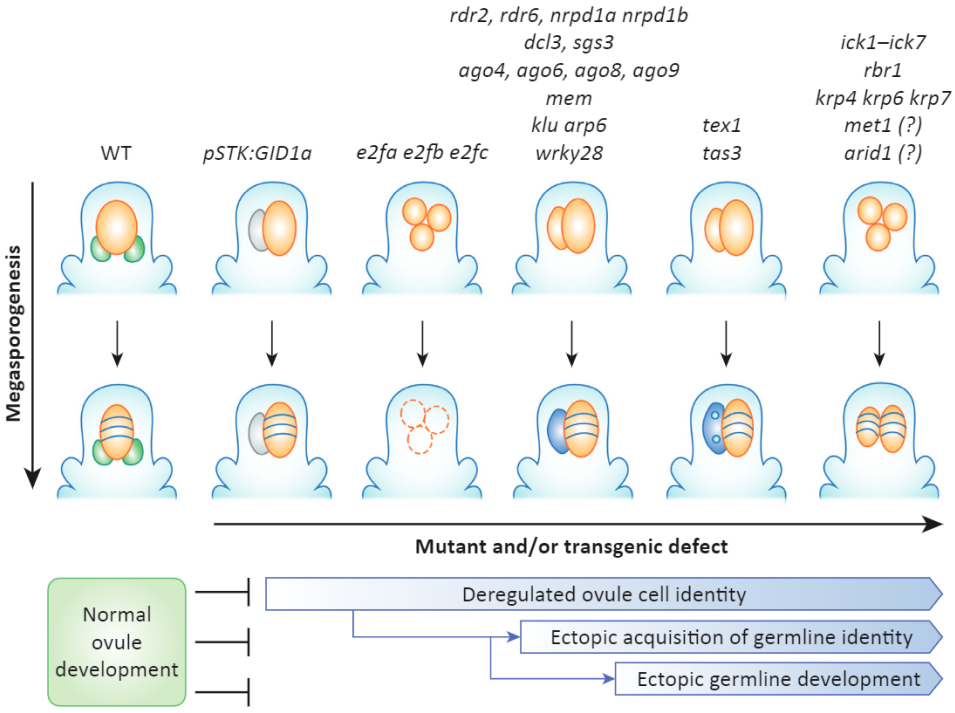

Figure 3. Ovule Mutants Reveal the Existence of Checkpoints That Define Distinct Stages of Germline Development. In the nucellus of young wild-type (WT) ovules, one germline cell, the megaspore mother cell (MMC), is specified (yellow oval) that in due course divides by meiosis (blue lines), while the neighboring nucellar cells (green) retain somatic identity. The first checkpoint restricts germline identity to the MMC, and possibly relates to a mechanism that recognizes or promotes cell expansion [5]. Specific cues appear to be required for this step because in *pSTK:GID1a*-expressing ovules multiple subepidermal nucellus cells enlarge, but only one acquires germline identity while the remainder do not develop further (grey oval) [33]. The second checkpoint regulates the acquisition of germline identity. In addition to morphological differences (Box 1), the germline exhibits a characteristic genetic program and epigenetic state [6,24] that defines the MMC and subsequently the functional megaspore (FM). Mutants in the RdDM pathway such as *nrpd1a/nrpd1b*, *rdr2*, *rdr6*, *ago4*, *ago6*, *ago8*, *ago9*, *dcl3*, and *sgs3* [15] (reviewed in [41]), *klu arp6*, and *wrky28* [28] fail to restrict germline identity, and additional subepidermal nucellus cells show MMC, female gametophyte (FG, blue oval), or mixed identity. The ability of these extra cells to continue germline development varies depending on the mutant. In *mem*, *tex1*, and *tas3* mutants the subepidermal nucellus cells occasionally start dividing as a gametophyte [27,35]. A third checkpoint is in place to restrict mitotic divisions to the FM, and meiotic divisions to the MMC. In the cell-cycle mutant *e2fa e2fb e2fc*, multiple germline cells (yellow circles) develop, possibly from MMC mitosis, but further development was not reported (broken circles) [32]. In *ick1 ick2 ick3 ick4 ick5 ick6 ick7* (*ick1–ick7*), *rbr1*, and *krp4 krp6 krp7* mutants, MMC meiosis is shifted towards mitosis, and germline progression is likely accomplished by formation of multiple FMs. The two FMs are derived from a single MMC that initially divides by mitosis [30,31]. It is possible that the same mechanism is disrupted in *arid1* and *met1* mutants because these appear to be morphologically similar to *rbr1* mutants [26]. The two rows of ovules show sequential stages of megasporogenesis in each mutant. The underlying flow chart groups the mutants into different classes based on (i) the apparent deregulation of ovule cell identity, and/or (ii) ectopic acquisition of germline identity, and/or (iii) continued ectopic germline development. Normal ovule development actively prevents this from taking place in the WT.

experiments determined that KLU and ARP6 are necessary for the deposition of H2A.Z near the *WRKY28* transcription start-site (TSS) [28]. To reconcile the different expression domains, the authors propose that the mobile signal generated by *KLU* diffuses into subepidermal nucellar cells to induce *WRKY28* transcription in a non-cell-autonomous manner (Figure 1D). Strikingly, specific expression of *WRKY28* in the MMC does not cause a shift in the MMC fate. Therefore, although *WRKY28* is necessary to prevent the subepidermal nucellar cells from acquiring MMC

identity, it does not control germline progression and is not sufficient to block germline fate in the MMC [28]. As was seen for the *ARP6–DMC1* pathway [25], this example supports the notion that specific mechanisms convey the change in fate from sporophytic to germline identity, while other factors allow progression of the germline program (Figure 3).

Defects in Cell-Cycle Machinery Deregulate the Germline Pathway

Perhaps unsurprisingly, differentiation of the germline and the subsequent acquisition of meiotic competence involve regulation of the cell cycle. Cyclin-dependent kinases (CDKs) promote G1- to S-phase transition by allowing the action of E2F TFs. On the other hand, KIP-RELATED PROTEINs (KRPs; also known as INHIBITORS OF CYCLIN-DEPENDENT KINASES, ICKs) inhibit cyclin-dependent kinases (CDK), and RETINOBLASTOMA-RELATED 1 (RBR1) directly blocks the action of E2F TFs (Figure 1D) [49]. Important insight regarding the *CDK*, *ICK/KRP*, *RBR1*, and *E2F* pathway comes from mammals, where homologous genes have an essential role in cell fate determination [50]. However, recent results from plants point towards several roles of this pathway during female germline development that appear to be distinct from its role in mammalian systems. Similarly to arabidopsis *rbr1* mutants, *krp4 krp6 krp7* mutants show ectopic MMC-like cells that appear to result from mitotic divisions of the MMC (Figure 3) [30]. Remarkably, these MMC-like cells can enter meiosis and develop into FGs. A similar phenotype was observed in the septuple *ick1 ick2 ick3 ick4 ick5 ick6 ick7* mutant, where >80% of ovules show two to four MMC-like cells that enter and complete meiosis (Figure 3) [31]. The origin of the ectopic MMCs in the septuple mutant was not investigated, but may result from mitotic division of the MMC, as shown for *rbr1* (Figure 3) [30]. By contrast, the origin of multiple FGs is less clear and may in fact be attributed to survival of non-selected megaspores rather than to an additional FM from the supernumerary tetrads. This is based on the finding that *ICK4/KRP6* expression is detected in degenerating megaspores, some *ick* septuple mutant ovules contain only one MMC but appear to show altered megaspore survival, and megaspores from the supernumerary meiotic tetrads appear to degenerate [31].

Another puzzling result comes from *e2fa e2fb e2fc* triple mutants, which might be expected to have no effect on MMC development given that E2F genes are usually repressed by RBR1 (Figure 1D) [51]. Despite this, triple mutants produce ovules with extra MMC-like cells that stop developing abruptly and typically fail to produce a FG (Figure 3) [32]. The origin of these cells is unclear but, considering that E2F TFs promote the transition to S phase, it seems unlikely that their absence would lead to MMC mitosis as occurs in *rbr1* mutants. Taken together, these studies suggest that cell cycle-related genes are key regulators of female germline progression in arabidopsis. ICK/KRPs function in different aspects of the germline cell cycle, ranging from restricting MMC mitosis to influencing FM selection [31]. Perhaps more importantly, these studies show that repression of mitotic division *per se* does not appear to be the sole causal factor that promotes initiation of meiosis in the MMC.

Competency Factors Such as WUS Also Influence Germline Progression

One target of RBR1 that reveals some insight regarding cell competency to enter meiosis or mitosis is the *WUS* gene, that was briefly discussed in the previous sections. *WUS* has multiple functions in plant development, including a role in MMC formation, without ever being expressed in the germline lineage (Figure 1D) [30,52]. The absence of *WUS* expression in ovules leads to the premature arrest of MMC development [52], possibly via deregulation of the small WINDHOSE 1 and 2 (WIH1/2) peptides that act downstream of *WUS* (Figure 1D) [21]. Although *WUS* mRNA and protein are absent from the MMC, both gene products are abundant in the surrounding nucellar cells. In the *rbr1* and *krp4 krp6 krp7* mutants WUS is detected in the

supernumerary MMCs, suggesting that it may promote their mitotic division, whereas in wild-type plants its absence from the MMC may enable the initiation of meiosis. Despite this, transgenic MMC-specific *WUS* expression is insufficient to cue mitotic divisions [30]. This suggests that additional factors are necessary to confer a mitotic state upon the MMC, and these are usually repressed during germline initiation. Components of this pathway might include DNA METHYLTRANSFERASE 1 (MET1) and AT-RICH INTERACTING DOMAIN 1 (ARID1), which are part of a sRNA pathway that is necessary for transposable element silencing [53,54]. It was recently shown that both *met1* and *arid1* mutants produce ovules containing one or more enlarged subepidermal nucellar cells (Figure 3) [26]. Although the nature of these abnormal cells was not investigated, another study indicates that *RBR1* represses *MET1* in the egg and central cell of the FG [55]. Therefore, future studies might consider whether this genetic relationship is maintained in the MMC (Figure 1D).

Species That Show Natural Deviations in Germline Development Provide Insight Regarding Pathways Controlling Cell Identity

From a historical perspective, much of the initial work on germline development focused on finding genes or pathways potentially involved in **apomixis**, a form of asexual reproduction that results in clonal maternal progeny through seed [56]. In apomictic species the alternation between haploid and diploid generations is avoided; unreduced FGs are either produced directly from the MMC (**diplospory**) or from somatic cells adjoining the megaspores (**apospory**), which bypass meiosis altogether. When combined with additional traits such as parthenogenesis and autonomous endosperm [57], unreduced female gametes facilitate the fertilization-independent production of clonal maternal progeny through seed ([58] for recent review).

Previous studies in arabidopsis have successfully recruited genes involved in meiosis to alter meiotic division in the MMC and produce unreduced gametes [59,60]. These pathways have been reviewed previously [57] and exhibit some features reminiscent of diplospory. These findings are complemented by recent studies reviewed here that highlight remarkable alterations in 'germline' development similar those seen in apomictic species. The two broad classes of arabidopsis germline mutants include those that produce extra FM/FG-like cells from somatic precursors (i.e., mutants in the RdDM, tasiRNA biogenesis, or chromatin-remodeling pathways) and those that appear to be derived from altered 'mitotic' MMC divisions (i.e., cell-cycle mutants), which mimic some of the differences seen between apospory and diplospory (Figure 3). Comparative transcriptomic studies tend to support this concept of distinct pathways underlying the two apomictic reproductive modes [61,62]. For example, in germline-like aposporous initials from *Hieracium praealtum*, key meiosis-related genes are not active [63,64], while in diplosporous species such as *Boechera gunnisoniana* the core meiotic genes are expressed [61]. This supports the notion that diplospory results from deviation of the meiotic pathway, whereas in apospory ectopic FM identity is established as a direct change in somatic fate [61].

Whether orthologous genes from natural apomicts share similar functions to the arabidopsis candidates described here remains to be determined; one caveat is that the loci controlling the initiation of apomixis are typically dominant and, in the case of apospory at least, appear to superimpose apomixis on a normal sexual program [65,66]. Hence, in aposporous apomicts, germline fate should be acquired by a sexual MMC and at least one somatic aposporous initial, with both having some capacity to produce a germline, depending on cell survival [65]. On the other hand, in ovules of diplosporous apomicts, only one cell progresses as a germline cell, therefore diplospory directly replaces sexual germline development [67].

It is equally plausible that the mutations described above, which lead to modified germline development in arabidopsis, represent upstream or downstream components of other regulatory pathways of greater importance in natural apomicts. For example, the *GIBBERELLIN-INSENSITIVE DWARF 1* (*GID1*) gene, which encodes a receptor for the gibberellin (GA) phytohormone, was recently highlighted as a possible upstream component of apomixis in *Brachiaria brizantha*, a facultative aposporous apomict that can reproduce via sexual reproduction or apomixis [68]. The binding of GA to GID1 leads to transcriptional activation of GA-responsive genes through degradation of repressive DELLA proteins (Figure 1D) (reviewed in [69]). In sexual *B. brizantha* plants, *BbrizGID1* is expressed in the MMC, while in apomitic *B. brizantha* plants *BbrizGID1* is expressed in the nucellus and in the MMC before the initiation of apospory. In arabidopsis, *AtGID1a* is expressed only in the base of the integuments (Figure 1D). However, ectopic expression of AtGID1a in the nucellus via the *STK* promoter leads to the formation of ovules with enlarged subepidermal nucellar cells (Figure 3), resembling *ago9* and *rdr6* mutants. While the abnormal nucellar cells do not express an MMC identity marker and fail to undergo meiosis, they also fail to initiate FG development. Hence, the GA–*GID1* pathway may contribute to nucellar cell identity or cell enlargement, but is insufficient to confer germline fate through ectopic expression in nucellar cells [33]. Components of GA signaling show expression profiles in the integument and nucellus during early ovule development in arabidopsis, but have mainly been implicated in integument development [70]. The role of these components appears to require further study, particularly in the context of apomictic versus sexual reproduction.

Concluding Remarks and Future Perspectives

In recent times the plant development community has revisited the process of female germline development. Given the location and size of the cells involved, the amount of detail that is now available is remarkable and it has clearly re-established the ovule as a model to investigate mechanisms controlling cell fate (Figure 1D). Technical advances such as laser-assisted microdissection (LAM) coupled with deep sequencing have provided transcriptomes of the MMC [35] and nucellus/FM [16] in both sexual and apomictic [61,63] species. Moreover, RNA sequencing, chromatin immunoprecipitation/sequencing, and CRISPR have potentiated the discovery of new regulators and mechanisms controlling germline formation [28]. Markers for distinct germline cell types such as *KNUCKLES* (*KNU* [16,71]; Box 2) have facilitated functional studies of candidate genes and enabled questions of cell identity to be addressed [26,28,30–32]. Moreover, the discovery of epigenetic pathways, some of which appear to act non-cell-autonomously, has provided insight into cellular crosstalk and the requirement for distinct chromatin states during germline development (Figure 1) [6,15,22,72]. Coupled to the regulation of cell cycle and competency regulators (WUS), the ovule has suddenly become much more crowded.

To summarize the recent findings in simple terms, it appears that, in the nucellus, epigenetic and cell-cycle mechanisms act concertedly to both establish and restrict MMC identity, inhibit mitosis, and provide competency to enter meiosis. Genes such as *AGO9*, *RDR6*, *RDR2*, *DCL3*, *NRPD1a/b*, *ARF3*, and *WRKY28* prevent somatic cells from acquiring germline identity (Figure 3) [15,27,28], whereas other undefined genes, that might be expected to show a similar expression profile to *KNU* or *MEM*, confer MMC identity. Cell cycle-related pathways such as *RBR1–WUS* and *ICK/KRP* mediate the inhibition of mitosis within the germline, but factors promoting cell expansion and subsequent progression into meiosis (possibly in concert with DMC1, KLU, and ARP6) remain elusive. These studies emphasize the importance of temporal molecular checkpoints that allow a single somatic cell to become a germline precursor, and the MMC to progress into meiosis and commit to germline fate.

Outstanding Questions

What are the transient and enduring pathways that act within the female germline to ensure that it maintains a unique identity compared to surrounding cell types? How have these pathways changed during evolution?

What are the mechanisms that modify cell architecture and facilitate germline cell expansion that are not activated in most ovule cells?

Do all non-germline cell types within the ovule nucellus share a similar identity, or are there different subpopulations, and if so how do these cells interact to coordinate development of the female germline?

Why do some 'ectopic' germline cells in arabidopsis ovule mutants enter meiosis whereas others do not?

Can the study of asexual (apomictic) species clarify the processes responsible for cell fate determinacy in sexual species?

What new technologies will facilitate the discovery of molecules involved in somatic cell–germline cell (nucellus–MMC) crosstalk?

These checkpoints likely ensure the MMC has time to adopt a correct epigenetic state and genomic integrity (Figure 2).

Future studies may focus on these checkpoints, potentially through refinement of the stages of nucellus and MMC development (see also Outstanding Questions). Further details of how and when somatic cells perceive and respond to germline-inducing signals still need to be elucidated. In addition, studies should focus on the genes that balance mitosis and meiosis to fully understand how female germline plasticity can be achieved. Resorting to single-cell transcriptomic and CRISPR technologies may facilitate this quest for germline specification factors.

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